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13. ABSTRACT *(Maximum 200 words)*

We have shown that cell lines developed from metrizamide density gradient fractions of peripheral blood hypodense (BJA.060.22) and hyperdense (BJA.060.24) eosinophils significantly inhibited MCF-7 colony formation in a dose-dependent fashion with as much as 82% inhibition ocurring at the E:T ratio of 1000:1 (BJA.060.22) and 85% inhibition of MDA-MB-231 cells at the same E:T ratio. At 1000:1 E:T ratio, the hyperdense eosinophil cell line BJA.060.24 inhibited MCF-7 colony formation by half that of the hypodense line (44%) and MDA-MB-231 colony formation by 70%. This inhibition is partially due to IL-4 as anti-IL-4 significantly abbrogated the inhibitory activity of 24 hr eosinophil culture supernatants. Furthermore BJA.060.22 also bound tightly to MCF-7 multicellular tumor spheroids (MTS) and transmission EM analysis revealed the presence of peripheral blood eosinophils in the core of the tumor spheroid and within its spaces. MTS in co-culture with eosinophilic cell lines offer an excellent model system to clearly define a role for eosinophils in cancer and their upregulation by cytokines and other regulatory agents, both at the cellular and molecular levels.

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Paulette Turbeville Harris 7/26/99

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Table of Contents

	Page Number
Front Cover	1
Standard Form 298	2
~~Forward~~ Foreword	3
Table of Contents	4
Introduction	5
Body	5
Key Research Accomplishments	7
Reportable Outcomes	7
Conclusion	8
References	9
Appendices	10

5. INTRODUCTION

Eosinophils are prinicipally known for their role in anti-helminthic responses as well as their inflammatory activity in allergic and asthmatic conditions (1). When activated, eosinophils release an array of inflammatory mediators including cytokines (2), many of which are toxic to the surrounding tissues. Moreover eosinophils, once activated by cytokines/chemokines can produce them and hence establish an autocrine cycle there by creating a constitutive eosinophilic presence at inflammatory sites (3-5). Eosinophils have been found in breast cancer infiltrates (6), however their active role in cancer, has not been well studied. We hypothesize that infiltrating eosinophils become activated and release cytokines into tumor inflammatory milieu and that these cytokines along with other released mediators inhibit growth of the tumor. In this study we have utilized eosinophils from allergic and asthmatic individuals with mild to moderate eosinophilia and cell lines established from them to investigate MCF-7 and MDA-MB-231 breast tumor cell growth inhibition in eosinophi:tumor co-culture assays.

6. BODY:

Propagation of Cell Lines. To date all eight eosinophilic cell lines have been retrieved from storage at -160°C, cultured in RPMI medium supplemented with sodium pyruvate (1mM), non essential amino acids (1mM), penicillin/streptomycin (50 units/50ug/ml, respectively), gentamycin (50ug/ml) and 10% fetal bovine serum; and refrozen. At present we have data with four of the cell lines.

Growth Inhibition of MCF-7 and MDA-MB-231 tumor cells by Eosinophilic Cell Lines.
a) Monolayer. Tumor cells (both MCF-7 and MDA-MB-231) were seeded into T_{25} culture flasks at 3×10^5 cells/flask and incubated overnight, (16-24hr) at 37°C, 5% CO_2. Eosinophils were added at effector to target (E:T) ratios of 5:1 and 43:1 (for eosinophil hypodense line SD.031.22) and 5:1 and 14:1 (for eosinophilic hyperdense line SD.031.24) and the flasks were incubated for 3 to 4 days. Both cell lines SD.031.22 and SD.031.24 markedly inhibited both MCF-7 (figure1B,1C,1D and 1E, respectively); and MDA-MB-231 cells (figure 2B, 2C, 2D and 2E, respectively). Results were similar when a third cell line, BJA.060.22 was tested against both MCF-7 (Figure 3) and MDA-MB-231 (Figure 4) tumor cells at E:T ratios of 1:1, (B) 20:1, (C) and 40:1, (D). The effector cells were removed, the flasks washed with phosphate buffered saline then stained with hematoxylin and eosin. Data were captured by photomicrography. In figure 5, peripheral blood eosinophil subpopulations hypodense (5A) and hyperdense (5B) confirm preliminary data of eosiniphilic destruction of tumor cell growth, at E:T ratios as low as 2:1, (5B, 5C).
b) Colony Formation. MCF-7 tumor cells were seeded into the wells of a 6-well tissue culture plate at 500, 250, 100 and 50 cells/well to establish cloning efficiency and optimum concentration for the inhibition assay. The plates were incubated at 37C, 5% CO_2 for ten days, until the colonies were visible by the naked eye. The plates were harvested, washed with PBS then stained with hematoxylin and eosin, and counted manually. In figure 6 , the percent cloning efficiency for the four cell concentrations (triplicate experiments) were 50, 52, and 56% (500 cells); 60, 64, and 72% (250 cells);140, 98, and 78% (100 cells) and 35, 45, and 80% (50 cells). From these data we originally selected both 250 and 100 cells, based on the percent efficiency, however we presently seed only 100 cells because of the facility in counting the colonies manually. Figure 7 shows colony inhibition of

MCF-7 cells by peripheral blood hypodense and hyperdense (A and B) eosinophils respectively. At 100:1 E:T ratios, both eosinophil subpopulations inhibited MCF-7 tumor colony formation by 79% and 80%, respectively. Hypodense eosinophilic cell line BJA.060.22 (Figure 8A) inhibited MCF-7 colony formation in a dose dependent manner at effector to target ratios of 10:1, 100:1, and 1000:1 (29%, 66% and 82%, respectively). The hyperdense cell line (BJA.060.24) inhibited colony growth by 33-44% at all ratios (figure 8B). At 100:1 it required 10-fold more cells from the cell lines to inhibit colony growth by the same extent as did the fresh eosinophils. Against MDA-MB-231 tumor cells BJA.060.24 eosinophilic cell line (figure 9B) inhibited colony formation dose dependently (3%, 43% and 70% at 10:1, 100:1, and 1000:1, respectively), while at 1000:1 BJA.060.22 (Figure 9A) dramatically inhibited colony formation by 93%.

Cytokine Presence in 24-hr Eosinophil Supernatants. 24hr supernatants from peripheral blood eosinophil hypodense and hyperdense fractions 22 and 24 were evaluated by enzyme-linked immunoassay (ELISA) analysis using commercial kits. Interleukin-4 and TNFα were present in varying levels in all six individuals tested. (Table 1). IL-4 concentrations ranged from 0 to > 1000 pg/ml while TNFα concentrations, which were far less than IL-4, ranged from 10-224 pg/ml. GM-CSF was only found in donor 6 at 450 pg/ml, and IL-5 was absent in all 6 samples.

24 hr Supernatants Inhibit MCF-7 Colony Formation. Based on the above results we evaluated 24hr supernatants for inhibition of tumor colony formation (Figure 10). Hypodense eosinophil fractions 22 inhibited MCF-7 colony formation by 30-50%. This inhibition was abbrogated in all six of the samples when anti-IL-4 antibody was added to the tumor cells along with the supernatants. Supernatants from hyperdense eosinophil fractions (24) inhibited MCF-7 colony growth by 26-48%, and anti-IL-4 antibody abbrogated the activity in 4/6 of the samples.

Infiltration of MCF-7 Multicellular Tumor Spheroids (MTS) by Peripheral Blood Hypodense and Hyperdense Eosinophil Fractions and by Eosinophil Cell Line.

a. MTS Production. MCF-7 multicellular tumor spheroids were developed by slightly modifying the method of Yuhas et al (7). Briefly, subconfluent monolayer cultures which were maintained (at 100% relative humidity, 95% air, 5% CO_2 at 37°C) in 10% RPMI complete medium . After trypsinization and cell count, the cells were dispensed into T_{25} non-vented flasks (1×10^6 cells/flask) and rocked at 30 rpm at 37°C for 24- 48hr, after which time the spheroids were transferred to 100 mm petri dishes containing an overlayer of 0.3% noble agar in 10% RPMI medium. The dishes were then incubated at 37°C in a 5% CO_2 atmosphere for 7-14 days with regular feeding to study the growth characteristics of the MTS.

b. Eosinophil: MTS Co-culture Assay. As early as 48hrs, thirty MTS were transferred to a fresh petri dish with agar overlay. Eosinophils were added at ratios of 10:1 and 100:1 eosinophil: MTS. The dishes were incubated for 7 days, then observed microscopically for eosinophil attachment. MTS were also fixed in 4% glutaraldehyde and processed with en bloc uramyl acetate for electron microscopic analyses.

Fig (11) shows photomicrographs of MTS in culture for 7 days. They range in size from 100 mm to 500 mm. Characteristic necrotic cores can be seen in A and C. Photomicrographs in figure (12) show fresh eosinophils, hypodense (A,C and E) and hyperdense fractions (B,Dand F) bound to spheroids, while in fig (13) eosinophil cell line BJA.060.22 is seen bound to the tumor spheroid. Transmission EM (Figure 14) analysis revealed eosinophils inside the MTS, both just inside the surface area and in the core. (Abstract to AACR)

7. Key Research Accomplishments

- Retrieval of all 8 eosinophilic cell lines
- Demonstration of functional cytotoxic/cytostatic activity with 4 of the lines
- Establishment of Multicellular Tumor Spheroids (MTS)
- Infiltration of MTS by eosinophils.

8. Reportable Outcomes

Hypodense and hyperdense eosinophils infiltrate MCF-7 breast multicellular tumor spheroids. Furbert-Harris PM, Harris D, Vaughn T, Parish-Gause D, Dunston GM, Abdelnaby A, Laniyan I, and Oredipe O. Proc Am Assoc Cancer Res. 40:455, 1999.

Furbert-Harris PM, Anderson D, Parish-Gause D, Vaughn T, Brown R, Laniyan I, Dunston GM, Abdelnaby A and Oredipe O.. Eosinophilic destruction of breast tumor cells in vitro is mediated by Interleukin-4. FASEB 13(4):A612, 1999.

9. Discussion/Conclusion

We hypothesized that activated eosinophils inhibit breast cancer cell growth by binding to the tumor cell, infiltrating the tumor and releasing inflammatory mediators, which include cytokines and these cytokines are know to have anti-cancer activity. The data presented have shown that subpopulations of hypodense and hyperdense activated eosinophils from individuals with mild to moderate eosinophilia inhibit the growth of MCF-7, and MDA-MB-231 breast tumor cells in vitro. Inhibition of colony formation was demonstrated with both activated eosinophils from peripheral blood as well as eosinophilic cell lines established from these hypodense and hyperdense subpopulations. At the effector to target ratio of 100:1, it required 10-fold more cells from the cell line to exact the same percent colony inhibition. This suggests that the fresh eosinophils are more activated than the cell lines and that perhaps treatment of these cell lines with cytokines will upregulate their level of activity. This is indeed one of the objectives of the study . Hence studies will be performed to treat cell lines with cytokines and other agents known to activate eosinophils. (e.g. IL-3, IL-4, IL-5, Ca ionophore), and evaluate their functional activity. The data indicating the absence of IL-5 and GM-CSF in 24hr culture supernatants of activated eosinophils is not 100% credible. Perhaps this is due to technical problems with ELISA Kits, particularly at the wash step. We are also still trying to select a single vendor for the ELISA Kits. There could also have been a problem with the kits themselves. We will introduce RT-PCR analysis for cytokine mRNA levels to complement the ELISA studies.

When eosinophils (both fresh and cell line) were co-cultured with MCF-7 MTS, (a model of micrometastatic nodules), the cells bound to the surface of the spheroid. This was seen by light microscopy: and transmission electron microscopy revealed the presence of eosinophils (fresh) within the spheroid. We will include scanning EM will include EM anaysis of cell line infiltrated spheroids and also immunohistochemical analysis of the spheroids for the presence of cytokines and other eosinophil mediators (e.g. granular proteins). Moreover kinetic studies of eosinophil attachment and infiltration are needed.

The resistant line MBA-MB.231 does not grow very well under our culture conditions. Colony formation was difficult to obtain. Hence the data with this cell line may be equivocal. We therefore will select another resistant breast cell line for future studies.

These studies thus far, confirm the preliminary observations of eosinophil destruction of tumor cells in vitro. The multicellular tumor spheroid model, along with the eosinophil cell lines (we have further purified the cells by FACS sorting and are presently carrying out phenotypic analysis - manuscript in preparation) offer an exquisite model system for defining the role for eosinophls as anti-cancer effector agents and their upregulation by cytokines and other agents.

10. References

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11. Apendices.

Figure 1. Inhibition of MCF-7 Tumor Cell Growth by Eosinophilc Cell Lines

Figure 1. Legend

Figure 2. Inhibition of MDA-MB-231 Tumor Cell Growth by Eosinophilic Cell Lines

Figure 2. Legend

Figure 3. Inhibition of MCF-7 Tumor Cell Growth by Eosinophilic Cell Lines

Figure 3. Legend

Figure 4. Inhibition of MDA-MB-231 Tumor Cell by Eosinophilic Cell Line

Figure 4. Legend

Figure 5. Inhibition of MCF-7 Tumor Cell Growth by Peripheral Blood Eosinophils

Figure 5. Legend

Figure 6. Plating Efficiency of MCF-7 Tumor Cells

Figure 6. Legend

Figure 7. Growth Inhibition of MCF-7 Tumor Cell Colony Formation by Peripheral Blood Hypodense and Hyperdense Eosinophils

Figure 7. Legend

Figure 8. Growth Inhibition of Colony Formation by Eosinophilic Cell Lines

Figure 8. Legend

Figure 9. Inhibition of MDA-MB-231 Tumor Cell Colony Formation by Eosinophilic Cell Lines

Figure 9. Legend

Table 1. Cytokine Concentrations in 24hr Eosinophil Culture Supernatants (pg/ml)

Table 1. Legend

Figure 10. Inhibition of MCF-7 Colony Formation by 24-hr Eosinophil Supernatants

Figure 10. Legend

Figure 11. MCF-7 Multi-cellular Tumor Spheroids

Figure 11. Legend

Figure 12. Peripheral Blood Hypodense and Hyperdense Eosinophils Bind to MCF-7 Multi-cellular Tumor Spheriods

Figure 12. Legend

Figure 13. Hypodense Eosinophilic Cell Line Binds to MCF-7 Multi-cellular Tumor Spheriods

Figure 13. Legend

Figure 14. Eosinophil Infiltration of MCF-7 Multi-cellular Tumor Spheriod

Figure 14. Legend

Abstract to AACR '99

Abstract to FASEB '99

Fig. 1. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Lines

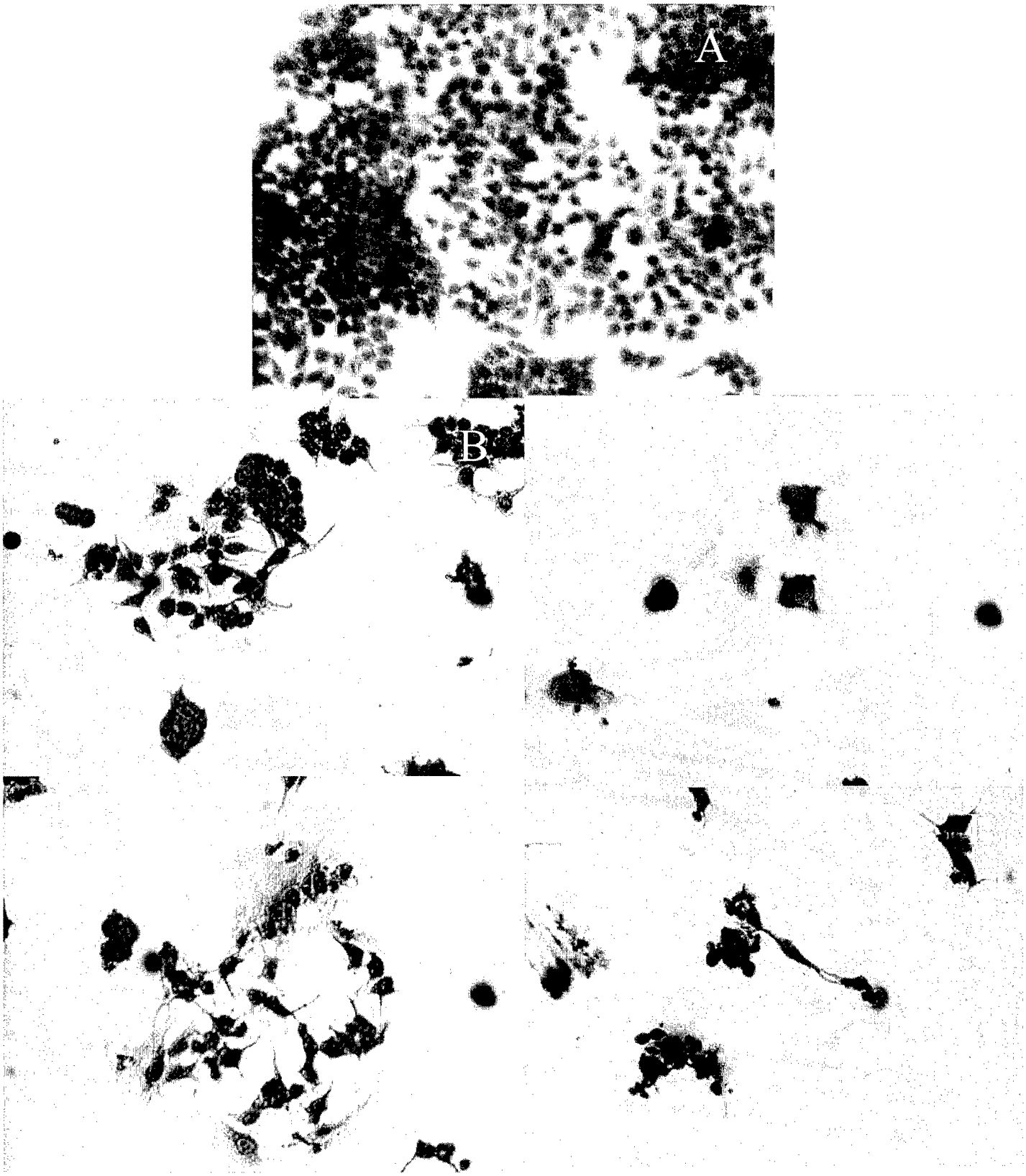

Fig. 1. MCF-7 tumor cells were seeded into T_{25} flasks at 3×10^5 cells/flask and allowed to grow to confluence (4-6 days) in media alone (A) or on the presence of hypodense eosinophilic cell line SD.031.22 at E:T ratios 5:l and 43:1 (B and C) and hyperdense - (D and E) cell line SD.031.24 at E:T ratios 5:l and l4:1.

Fig. 2. Inhibition of MDA-MB-231 Tumor Cell Growth by Eosinophil Cell Line

Fig. 2. MDA-MB-231 tumor cells were seeded similarly to MCF-7 in media (A) and with SD.031.22 (B) and SD. 031.24 (C) at similar E:T ratios 5:1, 43:1 and 5:1, 14:l, respectively.

Fig. 3. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Lines

Fig. 3. Hypodense eosinophil line BJA.060.22 was co-cultured with MCF-7 tumor cells similarly to SD.031.22, however the ratios were 1:1 (B), 1:20 (C) and 1:40 (D) control tumor cells were cultured in media alone (A).

Fig. 4. Inhibition of MDA-MB-231Tumor Cell Growth by Eosinophil Cell Lines

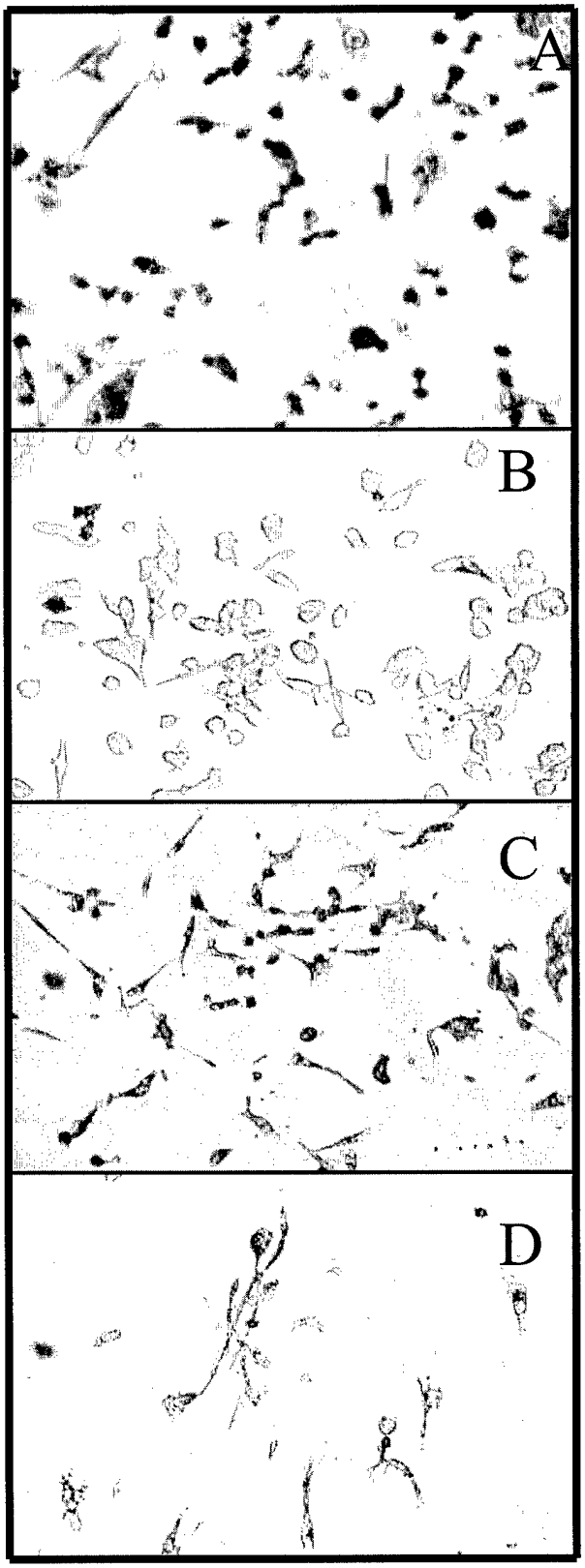

Fig. 4. Hyperdense BJA.060.24 was co-cultured with MDA-MB-231 tumor cells similarly to MCF-7 cells at E:T ratios l:1 (B), 20:1 (C) and 40:1 (D). Control tumor cells were cultured in media alone (A).

Fig. 5. Inhibition of MCF-7 Tumor Cell Growth by Peripheral Blood Eosinophils

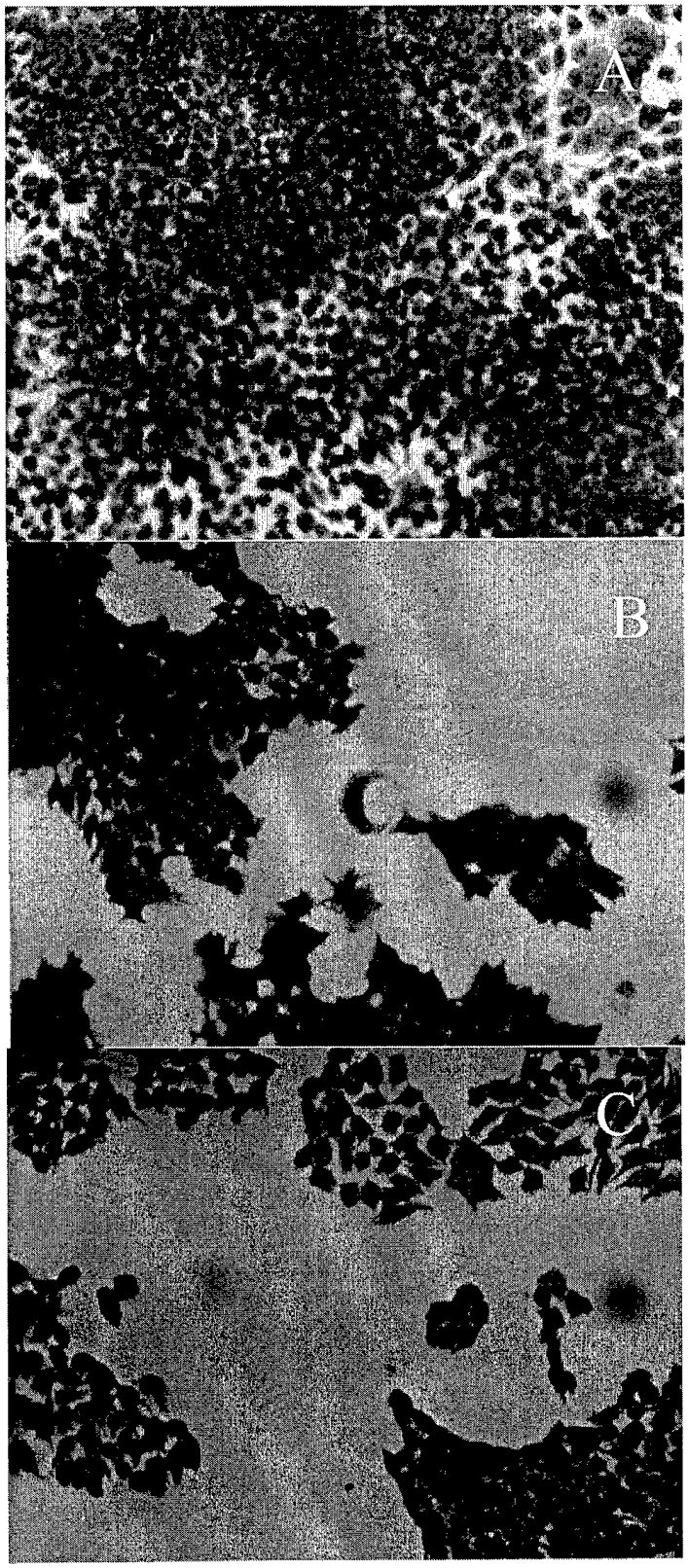

Fig. 5. Peripheral blood eosinophils, both hypodense (5B) and hyperdense (5C) show marked inhibition of cell growth when compared to that of the media control (5A) at 2:1 E:T ratios.

Fig. 6.

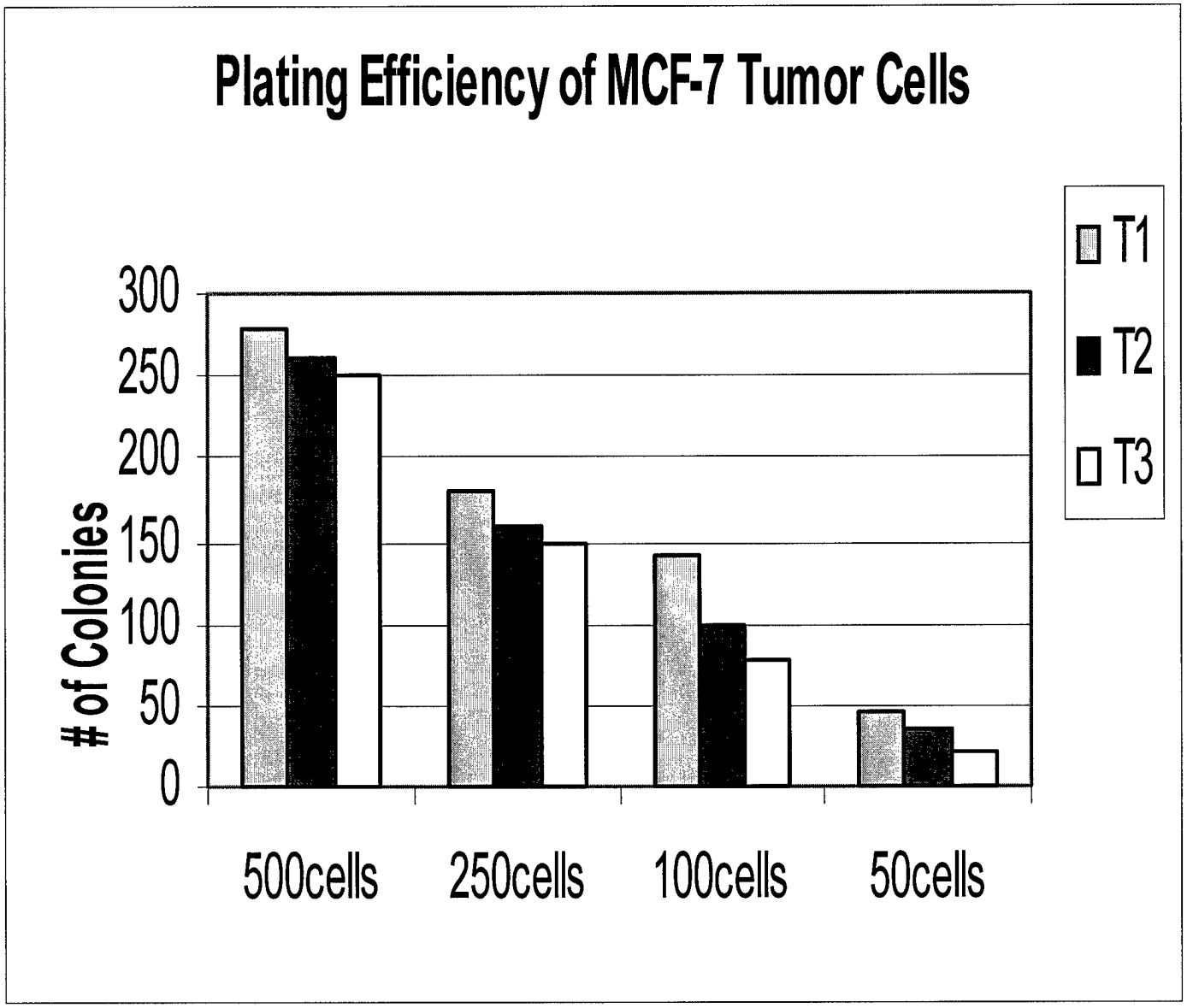
Plating Efficiency of MCF-7 Tumor Cells
# of Colonies
300
250
200
150
100
50
0
500cells
250cells
100cells
50cells
T1
T2
T3

Fig. 6. MCF-7 cells were seeded into 6-well plates at 500, 250, 100 and 50 cells per well. The plates were then incubated for 10 days at 37°C, 5% CO_2. Plates were washed 3X with PBS, then stained with hematoxylin and eosin.

Fig. 7. Growth Inhibition of MCF-7 Tumor Cell Colony Formation by Peripheral Blood Hypodense and Hyperdense Eosionphils

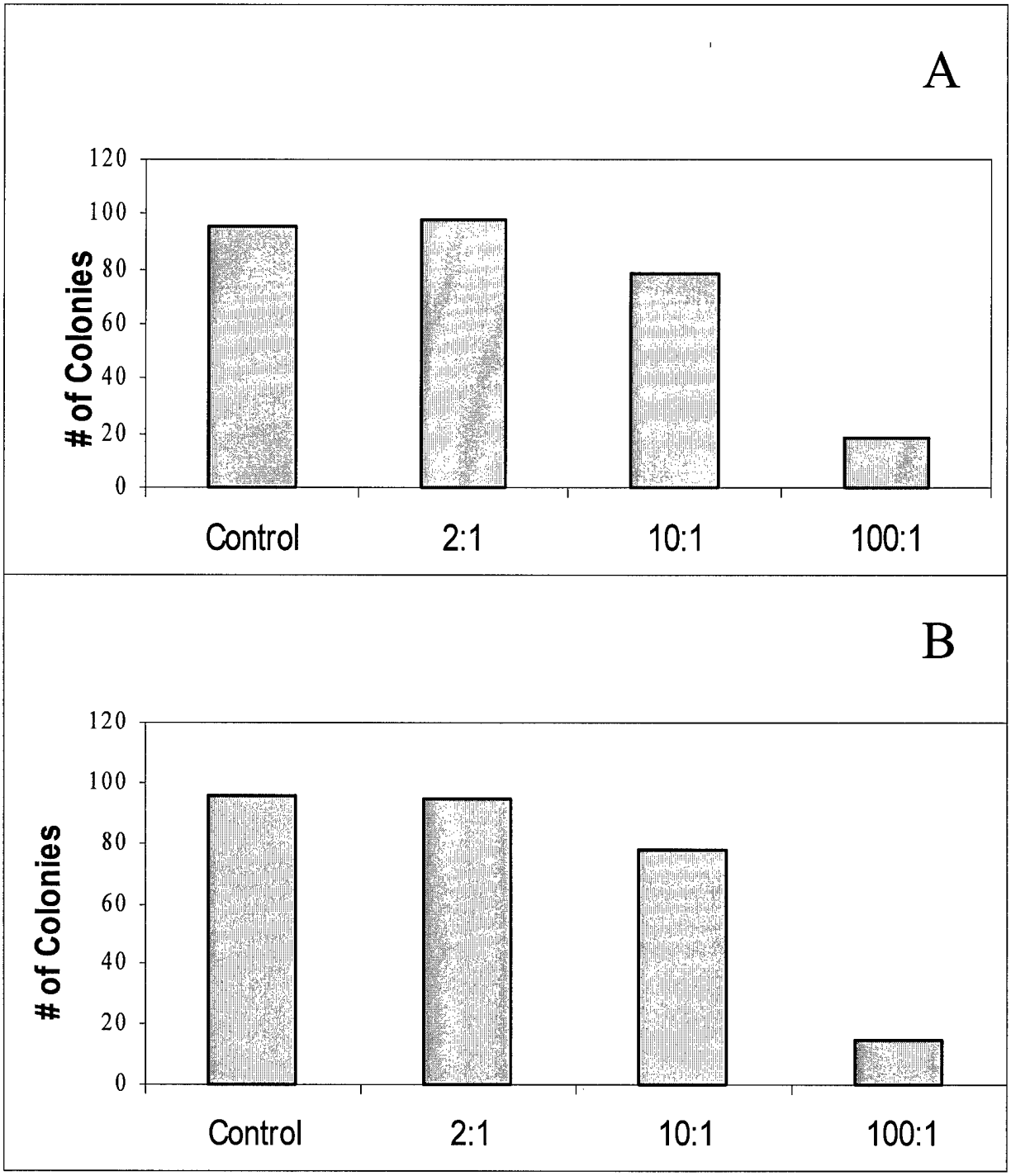

Fig. 7. MCF-7 cells were seeded into 6-well plates at 100 cells per well. 24hrs post seeding, hypodense (A) and hyperdense (B) eosinophils were added to the wells at E:T ratios of 2:l, 10:l and 100:1. All plates were incubated for 10 days at 37°C, 5% CO_2.

Fig. 8. Growth Inhibition of MCF-7 Colony Formation by Eosinophil Cell Lines

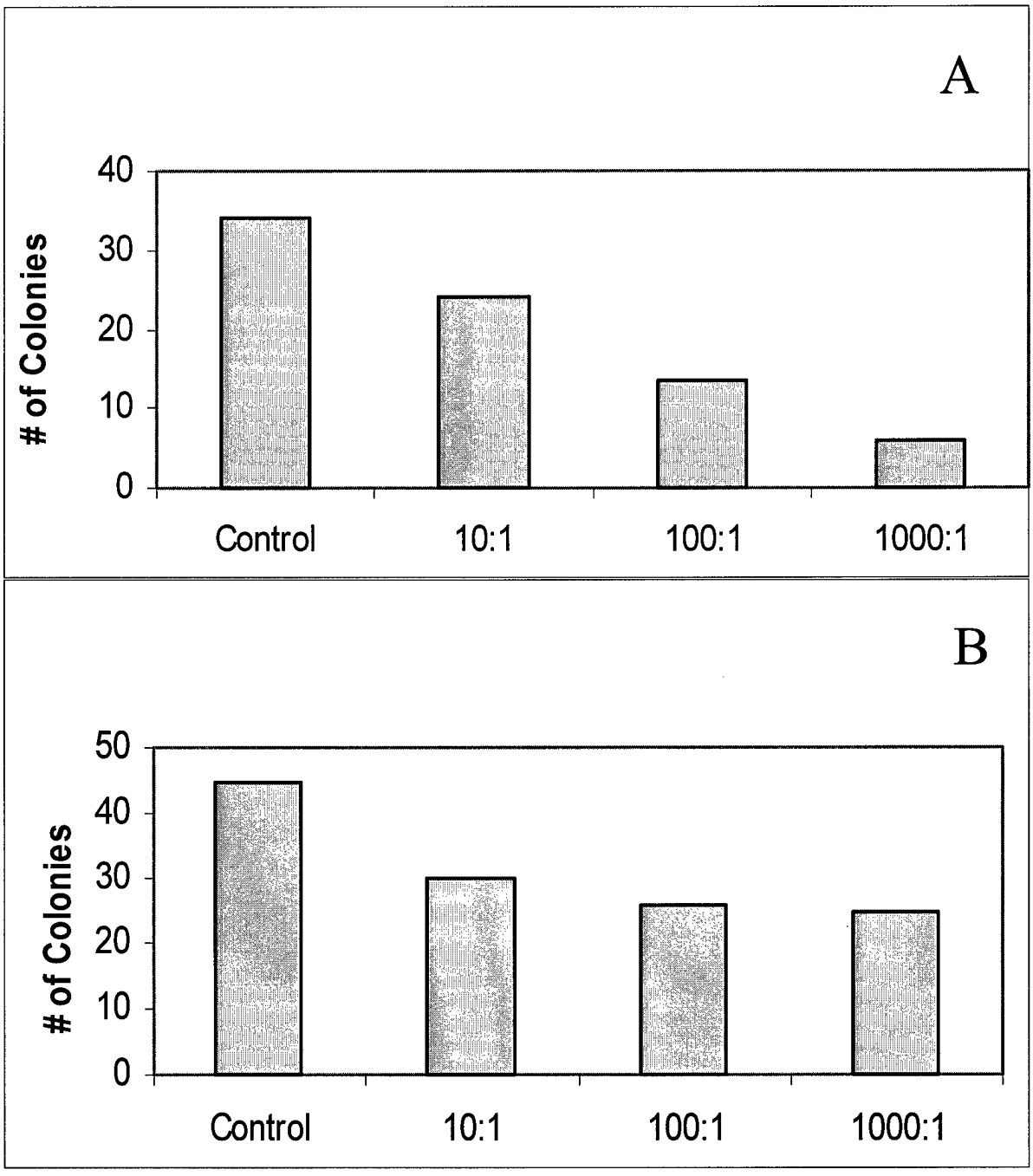

Fig. 8. BJA.060.22 (A) when cultured with MCF-7 cells at E:T ratios at 10:l, 100:l, and 1000:l inhibited colony formation dose dependently, while BJA.060.24 (B) did not.

Fig. 9. Growth Inhibition of MDA-MB-231 Tumor Cell Colony Formation by Eosinophil Cell Lines

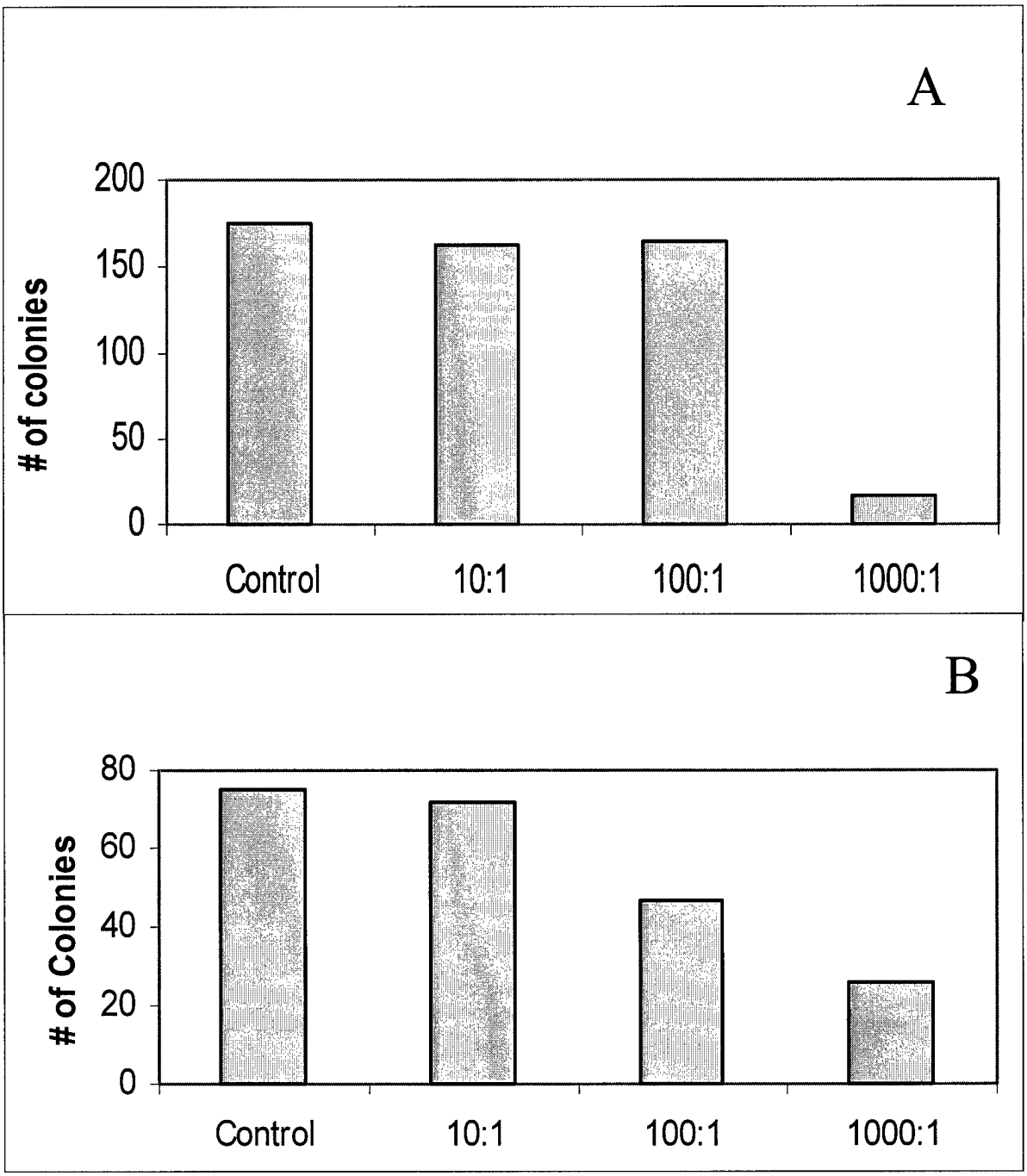

Fig. 9. BJA.060.24 (B) when cultured with MDA-MB-231 tumor cells at E:T ratios of 10:l, 100:l and 1000:1 inhibited colony formation in a dose dependent manner, while BJA.060.24 markedly inhibited colony formation (93%) at the E:T ratio of 1000:l.

Table 1. CYTOKINE CONCENTRATIONS IN 24HR EOSINOPHIL CULTURE SUPERNATANTS (pg/ml)

Donor	IL-4		IL-5		TNFα		GM-CSF	
	22	24	22	24	22	24	22	24
1	>1000	>1000	440	435	50	63	0	0
2	316	3	0	0	100	56	0	0
3	>1000	631	0	0	50	16	0	0
4	>1000	0	nt	nt	129	200	nt	nt
5	200	20	0	0	100	224	nt	nt
6	8	>1000	0	186	10	7.9	450	450

Table l. 24hr conditioned supernatants were tested for cytokines IL-4, IL-5, TNFα and GM-CSF using commercial enzyme linked immunoassay kits.

Fig. 10.

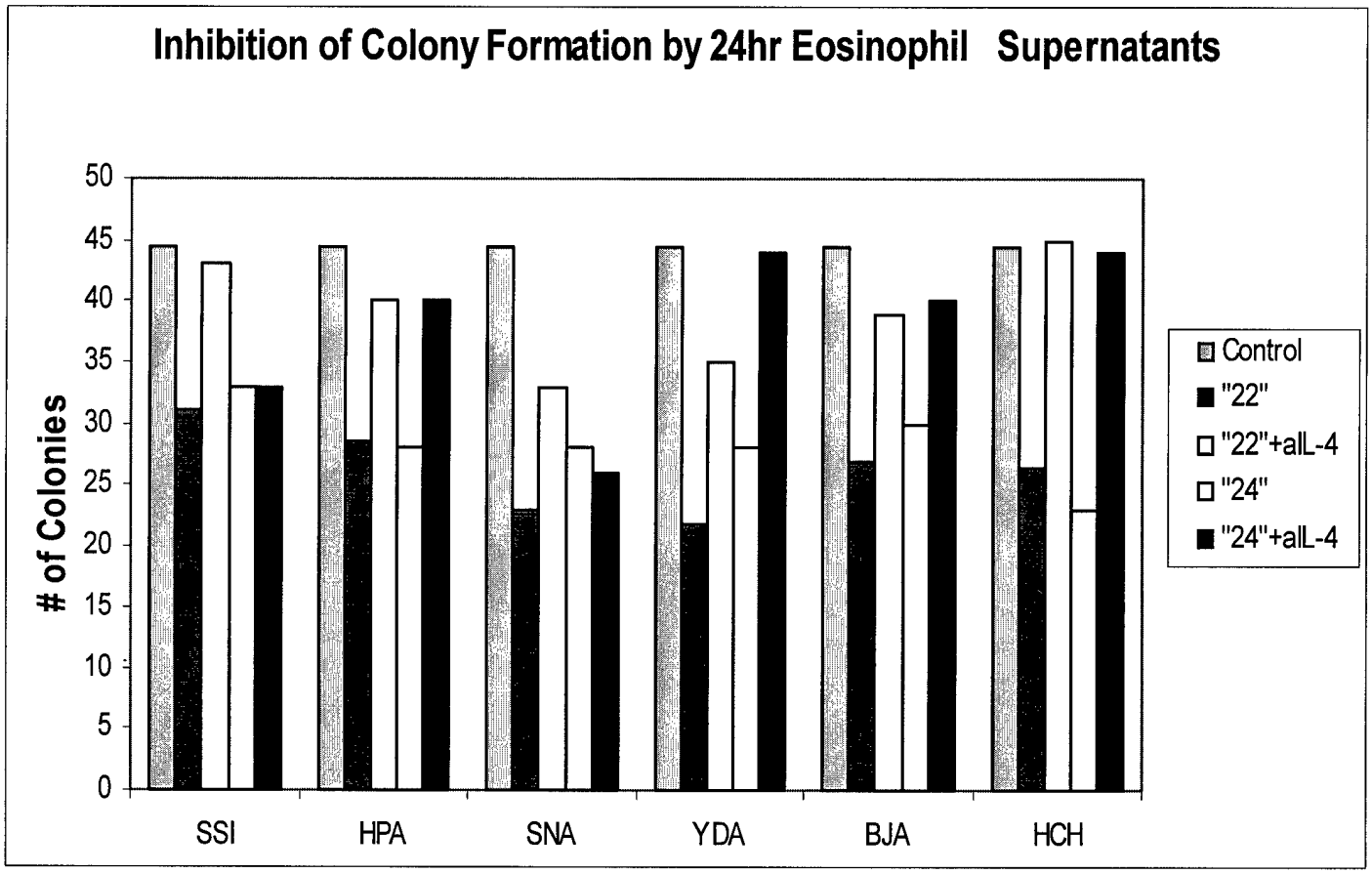
Inhibition of Colony Formation by 24hr Eosinophil Supernatants
# of Colonies
50
45
40
35
30
25
20
15
10
5
0
SSI
HPA
SNA
YDA
BJA
HCH
Control
"22"
"22"+aIL-4
"24"
"24"+aIL-4

Fig. 10. MCF-7 tumor cells, (100 cells/well) with media, conditioned supernatants or with anti-IL-4 and supernatants for 10 days afterwhich the cells were washed 3X with PBS, then stained with hematoxylin and eosin.

Fig. 11. MCF-7 Multicellular Tumor Spheriods

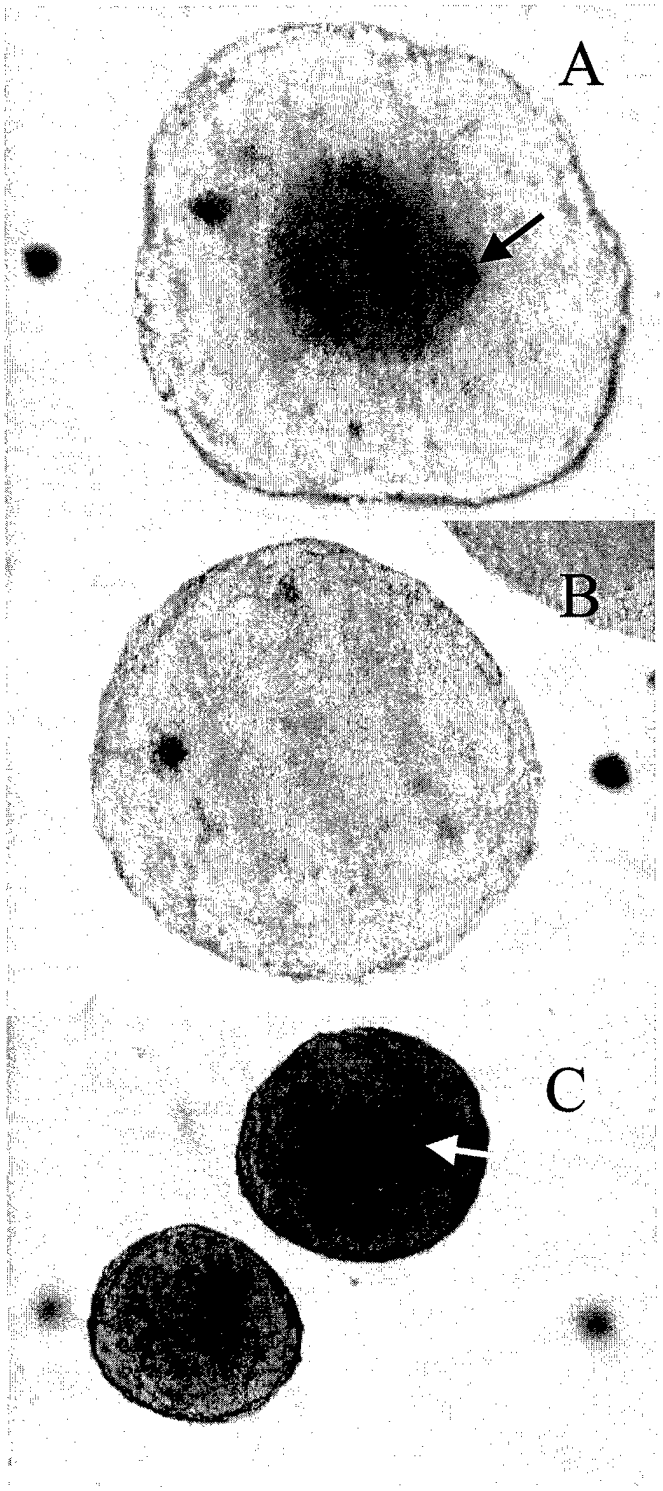

Fig. 11. MTS were developed by rocking 1×10^6 MCF-7 cells in a sealed T25 flask at 30 rpm for 48hr. At 37°C. The spheroids were then transferred to 100mm petri dishes containing 0.3% agar overlay, then cultured at 37°C, 5% CO_2 for 7-14 days.

Fig.12. Peripheral Blood Hypodense and Hyperdense Eosinophils Bind to MCF-7 Multi-cellular Tumor Spheriods

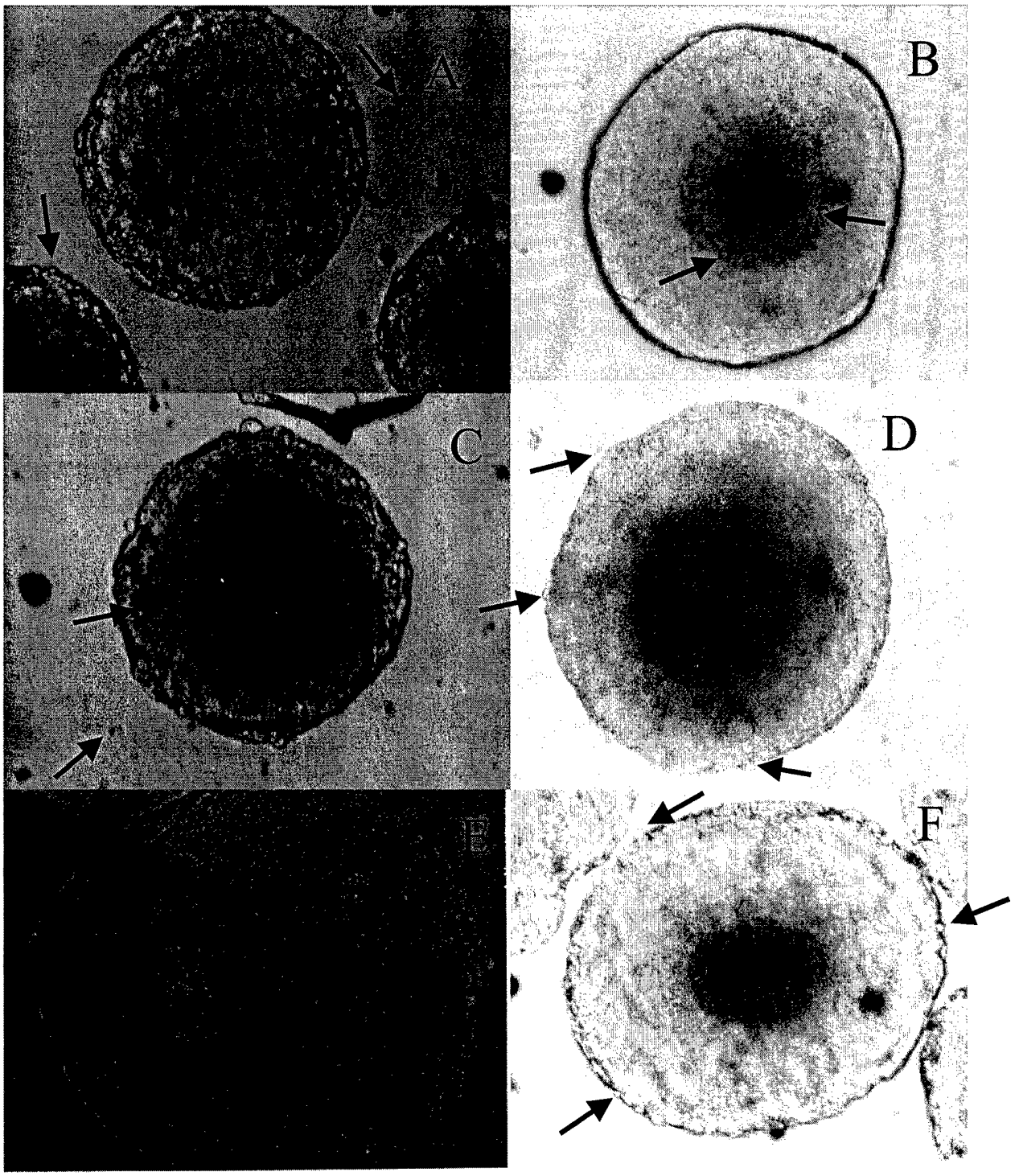

Fig. 12. Hypodense (A,C and E) and hyperdense (B,D and E) eosinophils from 3 individuals were cultured with 2 day-old MTS at an E:T ratio of 100:1 for 7 days. Arrows indicate bound eosinophils.

Fig. 13. Hypodense Eosinophilic Cell Line Binds to MCF-7 Multi-cellular Tumor Spheriods

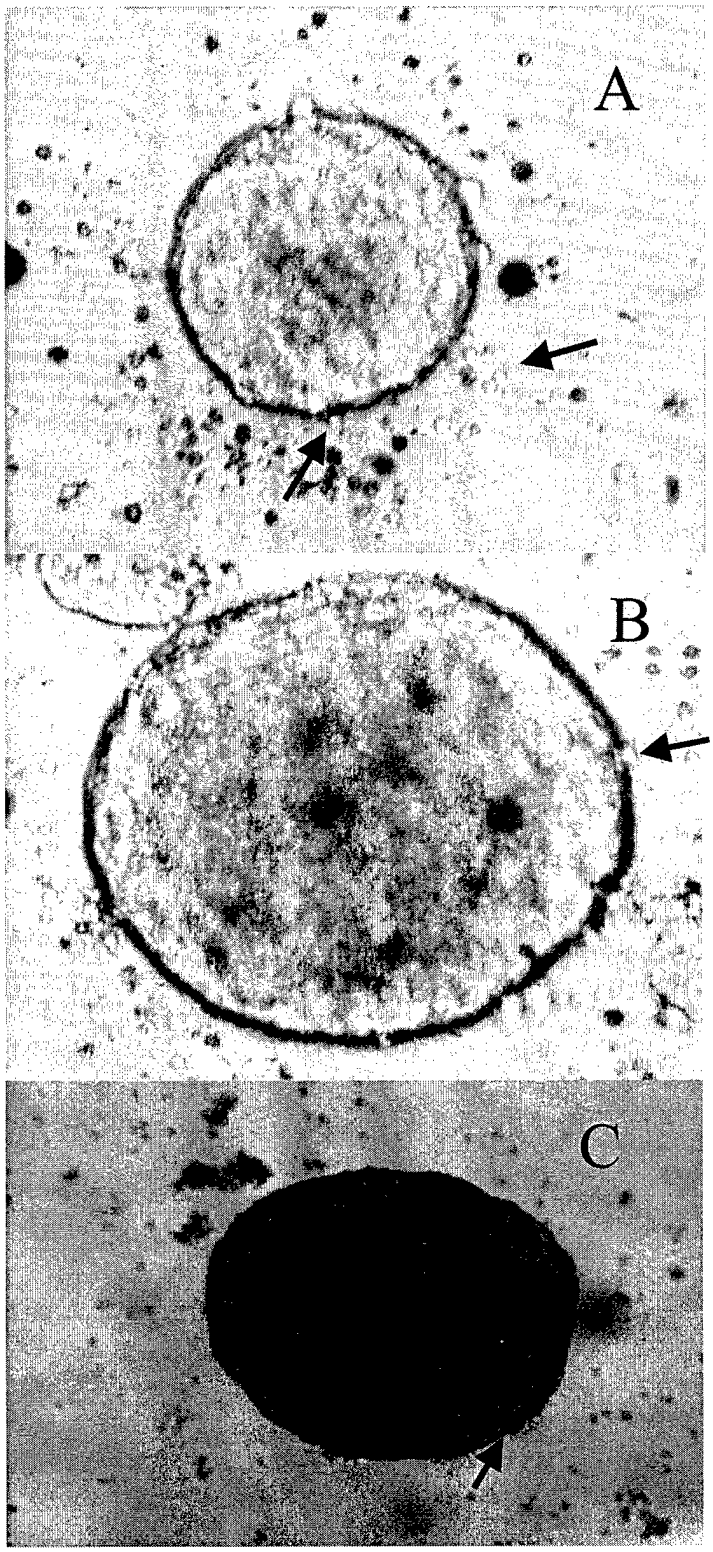

Fig. 13. Hypodense eosinophil cell line BJA.060.22 was cultured with 2-day old MCF-7 MTS at E:T ratios of 10:1 (A) 100:1 (B) and 1000:1 (C) for 7 days.

Fig. 14. Eosinophil Infiltration of MCF-7 Multicellular Tumor Spheroid

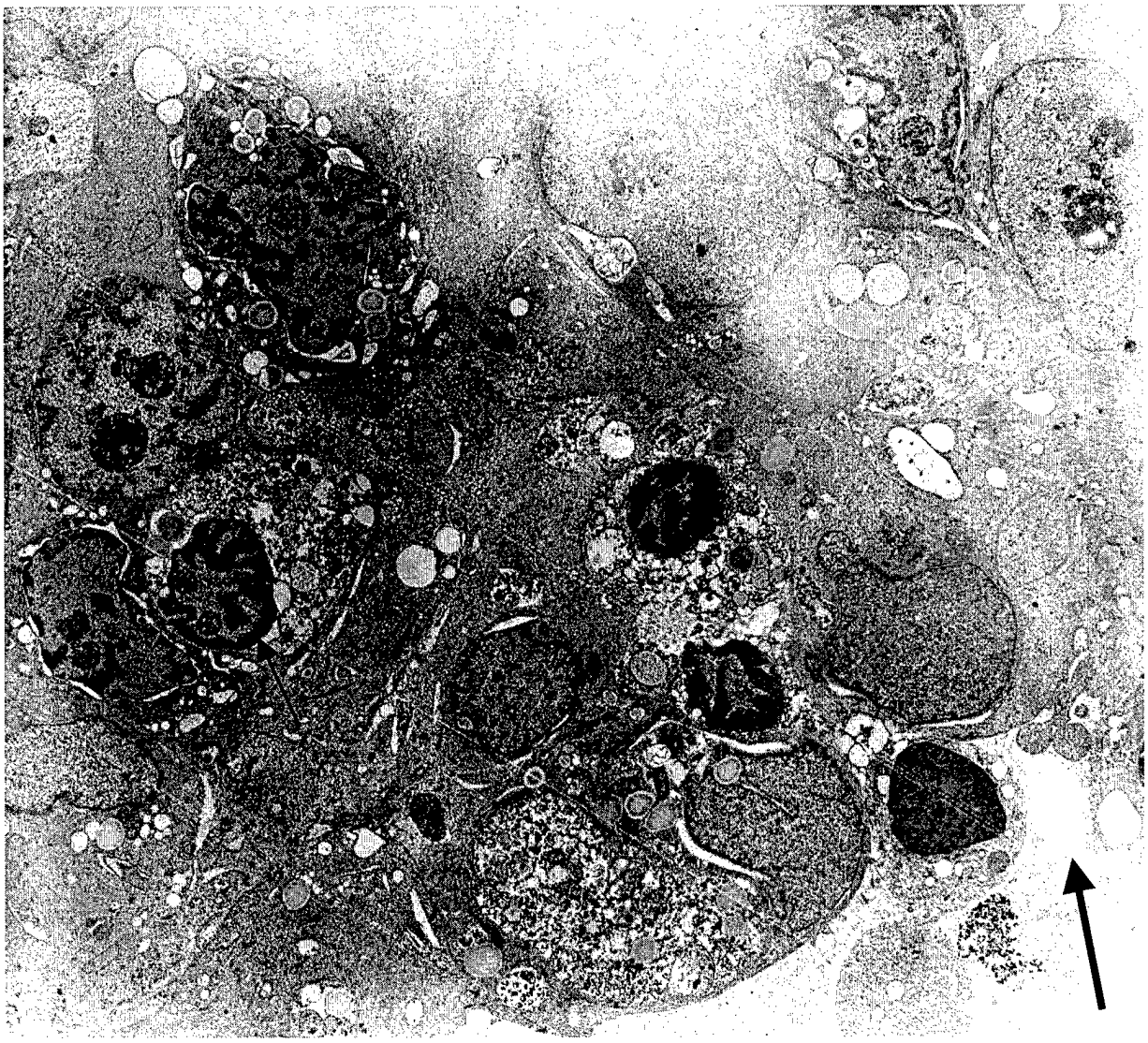

Fig. 14. Glutaraldehyde fixed tumor MTS:eosinophil complexes were prepared for transmission electron microscopic analysis. Eosinophils are seen in the MTS core (large arrow) as well within the spaces (small arrow).

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#3010 Hypodense and hyperdense eosinophils infiltrate MCF-7 breast multicellular tumor spheroids. Furbert-Harris PM, Harris D, Vaughn T, Parish-Gause D, Dunston GM, Abdelnaby A, Laniyan I, Oredipe O. *Howard Univ. Cancer Center, Wash., DC 20060.*

Our previous studies have indicated that activated eosinophils and eosinophilic cell lines inhibit *in vitro* growth of MCF-7 breast tumor cells. We have also shown that cytokines IL-4 and IL-5 also inhibit MCF-7 growth *in vitro*. In this study, MCF-7 multicellular tumor spheroids (MTS) were developed to study the effect of eosinophils, IL-4 and IL-5 on MTS growth. Hypo- and hyperdense metrizamide density gradient fractions of eosinophils from peripheral blood of individuals with mild to moderate eosinophilia were co-cultured with 2-day old MCF MTS in a medium containing bacto agar overlay in 5% CO_2 at 37C. Light microscopic analyses revealed attachment of effector cells to the spheroid borders. Moreover, the culture media was greater than 90% clear of effectors. At six days post co-culture, very large spheroids were observed in both test and control dishes; however the necrotic cores in the cocultures were more intense and larger than in the control. Furthermore, when MCF-7 tumor cells (1×10^6) were pretreated with IL-4 at 0.5 ng/ml, there was a dramatic decrease in the number of spheroids formed (90) compared to control (200). MTS are a more suitable model for *in vivo* development of micro metastatic nodules. These data strongly suggest that eosinophils are capable of infiltrating MTS and perhaps can release factors (cytokines like IL-4 and others which are capable of inhibiting tumor growth) into the extracellular spaces. These observations, along with the use of newly-established eosinophilic cell lines provide an exquisite model system for in depth studies of the role of eosinophils and cytokines in breast cancer and may offer potential therapeutic implications. D.O.D. #DAMD17-98-1-8107 and NIH# 5G12RR03048-12 RCMI Grant.

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ABSTRACTS
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Eosinophilic Destruction of Breast Tumor Cells In Vitro is Mediated by Interleukin-4. Furbert-Harris PM, Anderson D, Parish-Gause D, Vaughn T, Brown R, Laniyan I, Dunston GM, Abdelnaby A and Oredipe O.
Howard University, Washington, DC, 20060.

Previous studies in our laboratory have shown that activated eosinophils from individuals with eosinophilia will inhibit MCF-7 and MDA breast tumor cell growth in vitro. Moreover, eosinophilic cell lines established from these individuals inhibited MCF-7 colony formation in a dose dependent manner. In the present study, activated eosinophils were cultured for 24 hrs at 37C, 5% CO_2 and the conditioned media were captured for study. When MCF tumor cell monolayers were cultured with 24 hr supernatants, there was massive destruction of the monolayer similarly to that observed with the eosinophil-tumor co-cultures. MCF tumor cell colony formation was inhibited by both culture supernatants (37% and 31%, hypo- and hyperdense fractions, respectively) and by recombinant IL-4 (35%). Cytokine characterization of the supernatants by ELISA analysis indicated the presence of Interleukin-4 (IL-4) (> 1000ng/ml from both hypo- and normodense fractions) and TNFα (130 and 200 ng/ml from hypo- and hyperdense fractions, respectively). These results confirm the mouse data on eosinophil inhibition of tumor growth via IL-4 and strongly suggest a more active role for eosinophils as anticancer effectors. The regulation of this eosinophil anticancer activity by cytokines known to regulate eosinophils is under study. (Supported by DOD #DAMD17-98-1-8107 and NIH# 5G12RR03048-12 RCMI Grant)